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Serological classification and epitope specificity of *Proteus vulgaris* TG 251 from *Proteus* serogroup O65

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Abstract

Introduction: *Proteus* rods are currently subdivided into five named species, i.e. *Proteus mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri*, and *P. myxofaciens*, and three unnamed *Proteus* genomospecies 4 to 6. Based on the serospecificity of the lipopolysaccharide (LPS; O-antigen), strains of *P. mirabilis* and *P. vulgaris* were divided into 49 O-serogroups and 11 additional O-serogroups were proposed later. About 15 further O-serogroups have been proposed for the third medically important species, *P. penneri*. Here the serological classification of *P. vulgaris* strain TG 251, which does not belong to these serogroups, is reported. Serological investigations also allowed characterization of the epitope specificity of its LPS.
Materials and Methods: Purified LPSs from five *Proteus* strains were used as antigens in enzyme immunosorbent assay (EIA), SDS/PAGE, and Western blot and alkali-treated LPSs in the passive immunohemolysis (PIH) test, inhibition of PIH and EIA, and absorption of the rabbit polyclonal O-antisera with the respective LPS.
Results: The serological studies of *P. vulgaris* TG 251 LPS indicated the identity of its O-polysaccharide with that of *P. penneri* O65. The antibody specificities of *P. vulgaris* TG 251 and *P. penneri* O65 O-antisera, were described.
Conclusions: *P. vulgaris* TG 251 was classified to the *Proteus* O65 serogroup. Two disaccharide-associated epitopes present in *P. vulgaris* TG 251 and *P. penneri* O65 LPSs are suggested to be responsible for cross-reactions with three heterologous *Proteus* strains.

Key words: *Proteus*, lipopolysaccharide, O-serogroup, epitope, serological classification.

Abbreviations: EIA – enzyme immunosorbent assay, LPS – lipopolysaccharide, PAGE – polyacrylamide gel electrophoresis, PIH – passive immunohemolysis.

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INTRODUCTION

Much has been written about the taxonomy of *Proteus* since the original publication by Hauser, who established the genus, in 1885 [2]. Currently, the genus *Proteus* consists of five named species (*P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens*, and *P. hauseri*) and three unnamed *Proteus* genomospecies 4, 5 and 6 [6, 7]. *Proteus* rods are widespread in the environment and make up part of the normal flora of the human gastrointestinal tract. Bacteria of the genus *Proteus* are the third pathogen (after *Escherichia* and *Klebsiella*) which cause uncomplicated cystis, pyelonephritis, and prostatitis, particularly in hospital-acquired cases [12]. It was also suggested that *P. mirabilis* might play an etiopathogenic role in rheumatoid arthritis [16].

The serological specificity of *Proteus* strains is defined by the chemical structure of the O-specific polysaccharide chain (O-antigen) of the lipopolysaccharide (LPS, endotoxin). According to the serological specificity of the O antigens, strains of two species, *P. mirabilis* and *P. vulgaris,* were classified into 60 O-serogroups [5, 8], including 49 numbered serogroups (O1–O49) [5]. Fifteen further O-serogroups were proposed for the strains of *P. penneri* [3, 10, 17]. The aim of the present study was a serological classification of *Proteus vulgaris* strain TG 251, which does not belong to the Kauffmann-Perch scheme [5, 8]. Using serological and structural

data, the epitopes of *P. vulgaris* TG 251 LPS were investigated.

MATERIALS AND METHODS

Bacterial strains

Proteus vulgaris strain TG 251 was kindly provided by Prof. J. L. Penner (Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada). Thirty-nine *P. mirabilis* and 27 *P. vulgaris* strains were from the Czech National Collection of Type Cultures (National Institute of Public Health, Prague, Czech Republic). Twenty-four *P. penneri*, one *Proteus* genomospecies, and one *P. hauseri* strain were kindly provided by D. J. Brenner and C. M. O'Hara (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). A *P. myxofaciens* (CCUG 18769) strain was received from Dr. E. Falsen (Cultures Collection, University of Goeteborg (CCUG), Goeteborg, Sweden).

Cultivation of bacteria, isolation and saponification of the LPSs

Bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Łódź, Poland). LPSs were obtained by the extraction of dry bacterial mass with hot phenol/water [15] and purified with aqueous 50% trichloracetic acid at 4°C, followed by dialysis of the supernatant [19]. Alkali-treated LPSs were prepared by saponification of the LPSs with 0.25 M NaOH (56°C, 2 h), followed by precipitation with ethanol. The purified LPSs were used as antigens in the enzyme immunosorbent assay (EIA), SDS/polyacrylamide gel electrophoresis (SDS/PAGE), and Western blotting, and the alkali-treated LPSs in the passive immunohemolysis (PIH) test, inhibition of EIA and PIH, and absorption of the antisera with the respective LPSs.

Preparation of the O-polysaccharide

Delipidation of the *P. vulgaris* TG 251 LPS was performed by mild acid degradation with aqueous 2% HOAc at 100°C until precipitation of lipid A. The precipitate was removed by centrifugation (13,000×g, 20 min) and the supernatant was fractionated by gel-permeation chromatography on a column (56×2.6 cm) of Sephadex G-50 (S) (Amersham Biosciences, Sweden) in 0.05 M pyridinum acetate buffer (pH 4.5), monitored using a differential refractometer (Knauer, Germany). The yield of the high-molecular-mass O-specific polysaccharide of *P. vulgaris* TG 251 was 19% of the LPS weight.

NMR spectroscopy

The sample was freeze-dried twice from D_2O prior to measurement. ^{13}C-NMR spectrum was recorded with a Bruker DRX-500 spectrometer (Germany) for a solution in D_2O at 50°C using internal acetone (δ_C: 31.45) as reference. Standard Bruker software (XWINNMR 2.6) was used to acquire and process the NMR data.

Rabbit antisera and serological assays

The polyclonal O-antisera were obtained by immunization of New Zealand white rabbits with heat-inactivated bacteria of *P. vulgaris* TG 251 and *P. penneri* O65 according to the published procedure [18]. All serological tests were performed according to procedures described in detail elsewhere [11].

RESULTS AND DISCUSSION

In this paper we present the results of serological and immunochemical studies of LPS isolated from a *Proteus vulgaris* strain described in the Penner collection as TG 251 [8]. As judged by the essential identity of the ^{13}C-NMR spectra (Fig. 1), the O-specific polysaccharide isolated from the *P. vulgaris* TG 251 LPS has the same repeating unit as that of *Proteus penneri* 34 from serogroup O65, whose structure we determined earlier [14, 17] (Fig. 2). Therefore, in the serological investigations performed in this study, a second O-antiserum, that against *P. penneri* O65, was included. The LPSs of a number of *Proteus* strains with known O-polysaccharide structure were tested with polyclonal rabbit O-antisera against *P. vulgaris* TG 251 and *P. penneri* O65 by PIH and EIA to reveal possible serological relatedness of these bacteria.

Of 93 tested *Proteus* LPSs, including those of 39 *P. mirabilis*, 27 *P. vulgaris*, 24 *P. penneri*, one *P. hauseri*, one *P. myxofaciens*, and one *Proteus* genomospecies, only four strains were cross-reactive with *P. vulgaris* TG 251

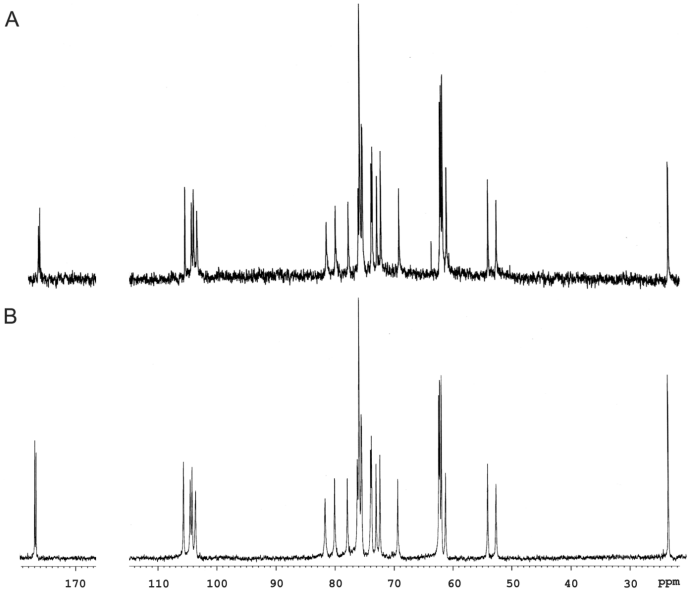
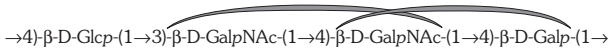
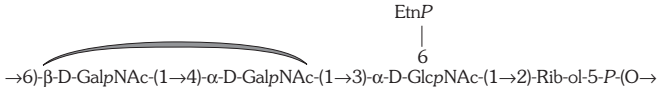


Fig. 1. ^{13}C-NMR spectra of the O-specific polysaccharides from the LPSs of *P. vulgaris* TG 251 (**A**) and *P. penneri* 34 (**B**).

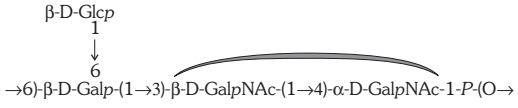
Proteus vulgaris O65 (TG 251)
Proteus penneri O65 (Pp 34) [1, 14, 17]



Proteus mirabilis O16 [13]



Proteus mirabilis O34 [9]



Proteus mirabilis O50 [4]

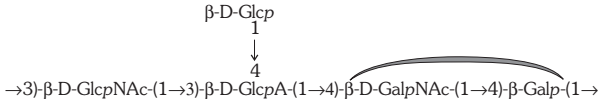


Fig. 2. Structures of the O-specific polysaccharides of cross-reactive *Proteus* LPSs. Tentative common epitopes are shown by arcs.

Fig. 3. Western blot of *Proteus* LPSs with O-antisera against *P. vulgaris* TG 251 (**A**) and *P. penneri* O65 (**B**). Pv, Pm, and Pp stand for *P. vulgaris*, *P. mirabilis*, and *P. penneri*, respectively.

O-antiserum, namely the LPSs of *P. mirabilis* O16, O34, and O50 and *P. penneri* O65. The strongest cross-reactivity, with *P. penneri* O65 LPS, was on the same level as with the homologous *P. vulgaris* LPS, thus suggesting again the close O-antigenic similarity of both strains. Based on this finding, *P. penneri* O65 O-antiserum was included in the subsequent detailed serological investigation, which involved PIH and EIA tests, inhibition of reactions in these tests, and absorption of O-antisera with the cross-reactive LPSs, followed by repeated PIH as well as Western blotting (Table 1, Fig. 3).

In PIH and EIA, both O-antisera reacted equally strongly with the homologous LPSs. A weaker reaction was observed with the heterologous *P. mirabilis* O16, O34, and O50 LPSs. Accordingly, the homologous LPSs were the strongest inhibitors of the reaction in both PIH and EIA, whereas the heterologous LPSs showed a weaker inhibitory activity. It is worth mentioning that *P. penneri* O65 O-antiserum cross-reacted more weakly with all heterologous LPSs in comparison with *P. vulgaris* TG 251 O-antiserum.

Table 1. Reactivity of O-antisera against *P. vulgaris* TG 251 and *P. penneri* O65 with the *Proteus* LPSs[a, b]

LPS from *Proteus* strains	Reciprocal titer for the LPS in		Minimal inhibitory dose (ng) of the LPS in	
	PIH	EIA	PIH	EIA
P. vulgaris TG 251 O-antiserum				
P. vulgaris TG 251	*51 200*	*512 000*	*1*	*2*
P. penneri O65	51 200	512 000	1	2
P. mirabilis O16	3 200	16 000	600	1 200
P. mirabilis O34	1 600	16 000	1 200	1 200
P. mirabilis O50	3 200	32 000	600	600
P. penneri O65 O-antiserum				
P. vulgaris TG 251	25 600	256 000	0.5	0.5
P. penneri O65	*25 600*	*256 000*	*0.5*	*0.5*
P. mirabilis O16	1 600	4 000	600	600
P. mirabilis O34	800	4 000	600	600
P. mirabilis O50	1 600	8 000	300	300

[a] LPS and alkali-treated LPS were used as antigens in EIA and PIH, respectively.
[b] Data for the homologous LPS are italicized.

In Western blot, both *P. vulgaris* TG 251 and *P. penneri* O65 O-antisera clearly recognized the same high--molecular-mass O-polysaccharide containing LPS species of both homologous strains, but the binding patterns of their low-molecular-mass LPS species, restricted to the core-lipid A moiety, were different (Fig. 3 A, B). All heterologous LPSs reacted significantly more weakly and bound only to high-molecular-mass LPS species.

The data obtained for the LPSs from *P. vulgaris* TG 251 and *P. penneri* O65 were essentially identical in all tests used. These results were in agreement with the structural investigation data of the *P. vulgaris* TG 251 O-specific polysaccharide, which revealed the same repeating unit as that of *P. penneri* O65, described in literature [14, 17]. They also support Penner's and Hennessy's conclusion [8] that *P. vulgaris* TG 251 does not belong to the Kauffmann and Perch scheme of the serological classification of *P. mirabilis* and *P. vulgaris* strains (which also does not include *P. penneri* strains). Therefore, we propose classifying the strain studied, *P. vulgaris* TG 251, to the *Proteus* O65 serogroup, in which *P. penneri* 34 (O65) has until now been the only representative.

Epitope characterization of P. vulgaris TG 251 LPS

In order to determine epitope specificity, the LPSs studied were tested using PIH with *P. vulgaris* TG 251 and *P. penneri* O65 O-antisera (Table 2). The reactivity of all the tested antigens was completely abolished when both O-antisera were absorbed with the homologous LPSs (Table 2). However, this absorption leaves a small fraction of antibodies in both O-antisera cross-reacting (1:1600) with the homologous LPSs of *P. vulgaris* TG 251 and *P. penneri* O65. These results could be accounted for by the difference in the LPS core region of the low-molecular-mass LPS species of these two strains shown in Western blot (Fig. 3). Absorption of both O--antisera with LPSs of *P. mirabilis* O16 and O34 removed antibodies cross-reacting to each other and decreased the reaction with the homologous antigens, but did not influence the reactivity with *P. mirabilis* O50 LPS.

A comparison of these serological results with the chemical structures of the O-specific polysaccharides of the cross-reacting LPSs described in literature [4, 9, 13] suggests that, in addition to the LPS core-specific antibodies, in both *P. vulgaris* TG 251 and *P. penneri* O65 O--antisera there are two more, major types of antibodies which recognize distinct epitopes in common with the LPS studied (Fig. 2). The first common epitope on the O-specific polysaccharides of *P. mirabilis* O16 and O34, responsible for their serological cross-reactions with both O-antisera, could be the β-D-Gal*p*NAc-(1→4)-β--D-Gal*p*NAc disaccharide fragment. This conclusion was confirmed by an absorption experiment (Table 2). Absorption with either of the heterologous LPSs (*P. mirabilis* O16 or O34) removed from both investigated O-antisera those antibodies which recognize the other strain from the pair and vice versa. Another independent fraction of antibodies in the investigated O-antisera seems to recognize the second common epitope associated with the β-D-Gal*p*NAc-(1→4)-β-D-Gal*p* disaccharide fragment shared by the homologous O-specific polysaccharides (those of *P. vulgaris* TG 251 and *P. penneri* O65) and that of *P. mirabilis* O50 (Fig. 2).

In conclusion, the serological results obtained in this study, combined with structural analysis data, demonstrated the identity of the O-specific polysaccharides from *P. vulgaris* TG 251 and *P. penneri* 34 (O65) LPSs

Table 2. Passive immunohemolysis data of the alkali-treated *Proteus* LPS with absorbed O-antisera against *P. vulgaris* TG 251 and *P. penneri* O65[a, b]

Alkali-treated LPS used for absorption	Reciprocal titer of absorbed O-antisera for the alkali-treated LPS from *Proteus* strains				
	P. vulgaris TG 251	*P. penneri* O65	*P. mirabilis*		
			O16	O34	O50
P. vulgaris TG 251 O-antiserum					
Control	51 200	51 200	3 200	1 600	3 200
P. vulgaris TG 251	*<100*	*<100*	*<100*	*<100*	*<100*
P. penneri O65	1 600	<100	<100	<100	<100
P. mirabilis O16	25 600	25 600	<100	<100	3 200
P. mirabilis O34	25 600	25 600	<100	<100	3 200
P. mirabilis O50	25 600	25 600	3 200	1 600	<100
P. penneri O65 O-antiserum					
Control	25 600	25 600	1 600	800	1 600
P. vulgaris TG 251	<100	1 600	<100	<100	<100
P. penneri O65	*<100*	*<100*	*<100*	*<100*	*<100*
P. mirabilis O16	12 800	12 800	<100	<100	1 600
P. mirabilis O34	12 800	12 800	<100	<100	1 600
P. mirabilis O50	12 800	12 800	1 600	800	<100

[a] Non-absorbed O-antisera were used as control.
[b] Data for the homologous LPSs are italicized.

and allowed classification of the former to the *Proteus* O65 serogroup, formerly represented by the single strain *P. penneri* 34. In O-antisera against both serogroup O65 strains studied, three different fractions of antibodies were detected and their LPS epitopes could be tentatively determined as follows. Major antibodies of the first type recognize the β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*NAc disaccharide as a common fragment present in the O-specific polysaccharides of *P. vulgaris* TG 251, *P. penneri* O65, and *P. mirabilis* O16 and O34 LPSs. Major antibodies of the second type bind to the β-D-Gal*p*NAc(1→4)-β-D-Gal*p* disaccharide fragment shared by the O-specific polysaccharides of *P. vulgaris* TG 251, *P. penneri* O65, and *P. mirabilis* O55 LPSs. Antibodies of the third type react with epitope(s) localized on the LPS core of *P. vulgaris* TG 251 and *P. penneri* O65 LPSs, whose nature as well as whole core structures remain unknown.

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